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Comparative study of mouse brains infected with Japanese encephalitis virus by intracerebral or intraperitoneal inoculation

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Summary. The brains of mice infected with Japanese encephalitis (JE) virus by intracerebral inoculation (IC), intraperitoneal inoculation with sham intracerebral inoculation (IP + sIC), and intraperitoneal inoculation (IP) were studied by light and electron microscopy. The mortality rates and mean survival days were 100% and 4.8 days for the IC group, 92% and 9.0 days for the IP + sIC group, and 58% and 13.4 days for the IP group. Accordingly, the brain samples of sick mice were examined by light and electron microscopy 4 days post-inoculation (p.i.) for the IC group, 7 days p.i. for the IP + sIC group and 12 days p.i. for the IP group. In light microscopy, the mouse brains in the IC group showed little inflammatory change with only mild generalized glial-cell proliferation and mononuclear cell infiltration. In electron microscopy, however, a majority of neurons in the brain were seen to be infected with virus that replicated exclusively in the neuronal secretory system, including rough endoplasmic reticulum (RER) and the Golgi apparatus. In contrast, light microscopic observation of the brains from the IP + sIC and the IP groups showed prominent inflammatory changes with leucocytic infiltration and perivascular cuffing. Neuronal degeneration and neuronophagia were also prominent. In electron microscopy, neurons were infected in the same manner as in the IC group, but showed more advanced degenerative changes with marked cytoplasmic rarefaction and frequent neuronal disintegration. Mononuclear cells were frequently found in direct contact with degenerating and disintegrating neurons. The results showed that (a) the basic process of JE virus replication in brain neurons was present in the three groups of mice, (b) in the peripherally inoculated mice the process was accompanied by inflammatory reaction with resultant neuronal destruction, and (c) breach in the blood–brain barrier at the time of peripheral viral inoculation played an important role in the viral invasion of the CNS.

Keywords: brain neuron, intracerebral and intraperitoneal inoculation, Japanese encephalitis, light and electron microscopy

The pathogenesis of Japanese encephalitis has been studied mainly in the animal model in which laboratory mice are infected with Japanese encephalitis (JE) virus by intracerebral inoculation. By this model, it has been shown that JE virus replicates in a majority of

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neurons in the central nervous system (CNS), causing death to the animals (Yasuzumi & Tsubo 1965; Oyanagi *et al.* 1969; Hase *et al.* 1990). Furthermore, recent electron microscopical observations have revealed that the virus grows exclusively in the neuronal secretory system including rough endoplasmic reticulum (RER) and the Golgi apparatus and, in the process, selectively destroys these cytoplasmic membranous organelles of infected neurons (Hase *et al.* 1990). Destruction of neurons themselves as the result of viral replication is not prominent; therefore, it is thought that functional insufficiency involving a large number of JE virus-infected neurons in the CNS eventually brings about the fatal outcome in the animals. This idea seems to be particularly relevant since neurons in the brain stem, including the lower pons and the medulla oblongata, are usually involved in the infection (Oyanagi *et al.* 1969; Hase *et al.* 1990).

One characteristic difference of the animal model from human Japanese encephalitis is that the histological examination of the CNS of intracerebrally inoculated mice reveals very little inflammatory change in contrast to that of fatal human cases in which prominent inflammatory lesions such as perivascular cuffing, leucocytic infiltration, neuronophagia, and glial nodule formation have been reported (Miyake 1964). As a matter of fact, because of strong emphasis on the occurrence of inflammatory lesions in human Japanese encephalitis, the relevance of the animal model to human Japanese encephalitis is in doubt. It is noted, however, that the animal model constitutes a very rapidly fatal infection in which mice may die before any appreciable host immunological response takes place in the brain. In contrast, even the most acutely fatal human cases take more than a week, counting the incubation and acute periods (Halstead 1981). The route of intracerebral inoculation also differs from that in natural infection in which the virus is introduced peripherally through the bite by vector mosquitoes.

Intraperitoneal inoculation of JE virus also causes death in a certain percentage of mice following a more prolonged disease course. In this respect, it is of interest to see whether the brains of mice infected by peripheral inoculation show pathological changes more closely related to those observed in human cases. In this study, therefore, we have compared the histological and electron microscopical observations of the brains of mice inoculated intracerebrally and intraperitoneally with JE virus to determine whether the duration of disease or the route of infection influences the occurrence of inflammatory changes in the brain.

Materials and methods

Virus

The SA_{14} strain of JE virus was obtained from Dr Yu Yong Xin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). After two passages in suckling mouse brains, a seed stock was prepared as 20% infected mouse brain homogenate at the third passage. Virus preparations were clarified by centrifugation at 600 *g*, aliquoted and stored at −70°C.

Mouse infection

Young adult mice (ICR strain, 4–5 weeks old) were used. Infectivity titres (LD_{50}) were determined in mice, using the intracerebral route of inoculation, and were calculated by the method of Reed and Muench (1938). For observation and microscopical studies, mice were inoculated with 640 mouse LD_{50}. The IC group received 0.03 ml of the inoculum intracerebrally; the IP+sIC group received 0.3 ml of the inoculum intraperitoneally and at the same time were injected intracerebrally with 0.03 ml of virus-free diluent (Eagle's minimal essential medium); and the IP group received 0.3 ml of the inoculum intraperitoneally. Surviving mice were observed daily for 21 days.

Light and electron microscopy

For light and electron microscopy, sick mice in the IC group were sacrificed 4 days p.i.; those in the IP+sIC group 7 days p.i.; and those in the IP group 12 days p.i. They were anaesthetized and perfused with 20 ml of phosphate buffered saline (PBS), followed by 20 ml of 4F1G fixative (McDowell & Trump 1976), by injecting the fluids from the left ventricle under a mild pressure and draining them from a cut in the right atrium. The perfused mice were placed in a refrigerator at 4°C for 30 min. The brain was excised and cut at coronal planes. Although the cerebral cortex, the basal ganglia–thalamic region, the cerebellum, and the medulla oblongata were routinely examined, the cerebral cortex taken at the coronal plane passing through the pituitary stalk was used for comparison in the study. Mice injected intracerebrally with 0.03 ml of the virus-free diluent were used as controls for all the groups. Before processing, the pieces of brain tissue were further fixed in 4F1G fixative at 4°C overnight. For light microscopy, the pieces of brain tissue were embedded in paraffin. Sections were cut at 5-μm thick and stained with haematoxylin and eosin. For electron microscopy, the brain tissue pieces fixed in 4F1G were washed in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% cacodylate-buffered osmium tetroxide, dehydrated, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss SM109 or Philips CM12 electron microscope.

Results

Mouse infection

The mortality rates and the mean survival days of the three groups of mice are shown in Table 1. The mice in the IC group usually became sick rather suddenly 4 days after inoculation and died abruptly on the following day with a mortality rate of 100%. The peripherally inoculated mice became less acutely ill, often developing clearly recognizable paralysis of the limbs. The mortality rate of the mice of the IP+sIC group was 92% with a mean survival of 9.0 days. The mortality rate of the IP group was 58% with a mean survival of 13.4 days.

Table 1. Observation of mice inoculated with JE virus by various routes of inoculation

Inoculation	Mean survival (days)	Mortality	
		Ratio	Percentage
IC	4.8	13/13	100
IP+sIC	9.0	12/13	92
IP	13.4	7/12	58

*Mice received 640 LD_{50} as described in Materials and methods.

Light microscopy

Representative histological pictures of the cerebral cortices of mice from the three groups are shown in Fig. 1. The cerebral cortex of the IC group showed some vascular engorgement and mild glial proliferation and mononuclear cell infiltration (Fig. 1B). Some oedema in the tissue was noticed, but inflammatory changes were minimal. In contrast, the cerebral cortices of the IP+sIC and IP groups showed inflammatory changes with leucocytic infiltration that involved both the leptomeninges and the brain tissue (Fig. 1C,D). There were prominent perivascular oedema and leucocytic infiltration that formed perivascular cuffing. In general, the inflammatory changes were more pronounced in the brains of the IP group than in those of the IP+sIC group. In higher magnification, histologic changes of neurons of the brain in the IC group were rather unremarkable (Fig. 2A). On the other hand, in the IP+sIC and IP groups, many neurons of the brains showed characteristic cytoplasmic rarefaction (Fig. 2B,C). The cytoplasm of

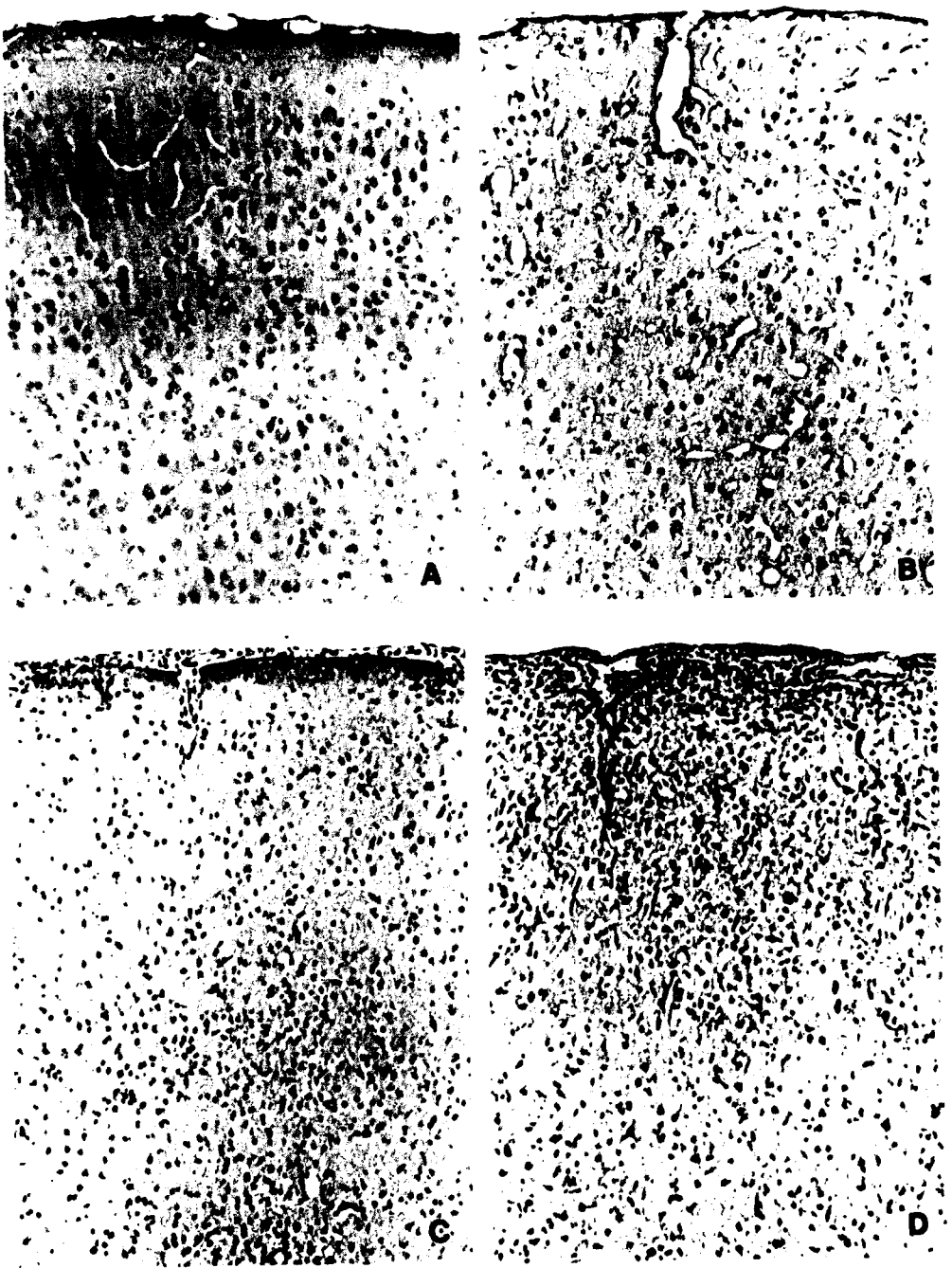

Fig. 1. Representative pictures of the cerebral cortices of mice in the three groups. A. Control. B. The IC group showing accentuation of capillaries and possible oedema. Mild glial proliferation and leucocytic infiltration may be present. C. The IP + sIC group showing leucocytic infiltration of the leptomeninges and the cerebral cortex. D. The IP group showing severe leucocytic infiltration of the leptomeninges and the cerebral cortex. × 100.

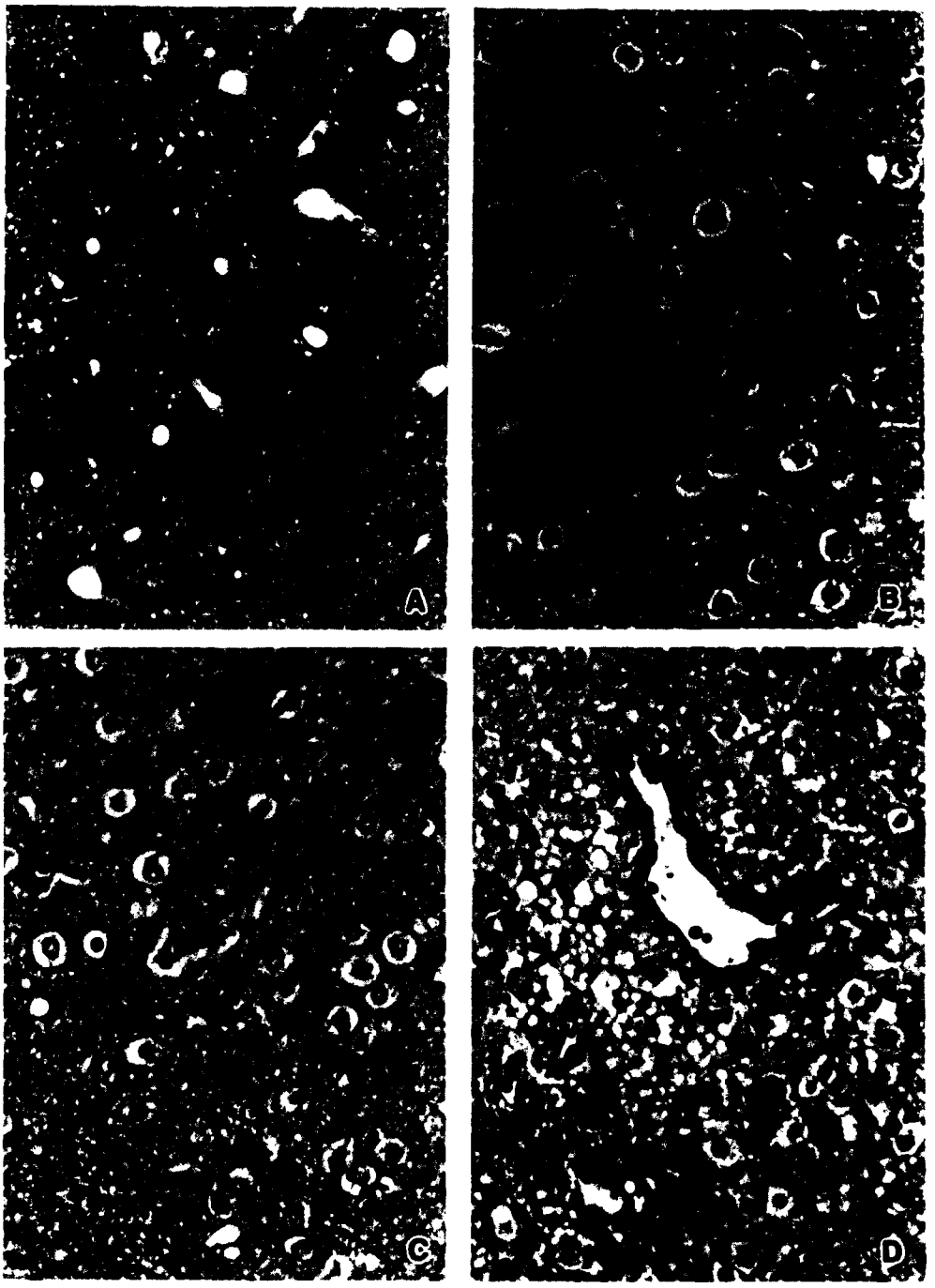

Fig. 2. Neuronal degeneration of the cerebral cortices of mice in the three groups. A. The IC group showing relatively little neuronal changes. B. The IP + sIC group showing cytoplasmic rarefaction and rounding of neurons. C. The IP group showing prominent cytoplasmic rarefaction and degeneration of neurons. D. The IP group showing perivascular oedema and leucocytic infiltration. Neurons in the adjacent area show degenerative changes with cytoplasmic rarefaction and nuclear pyknosis. × 250.

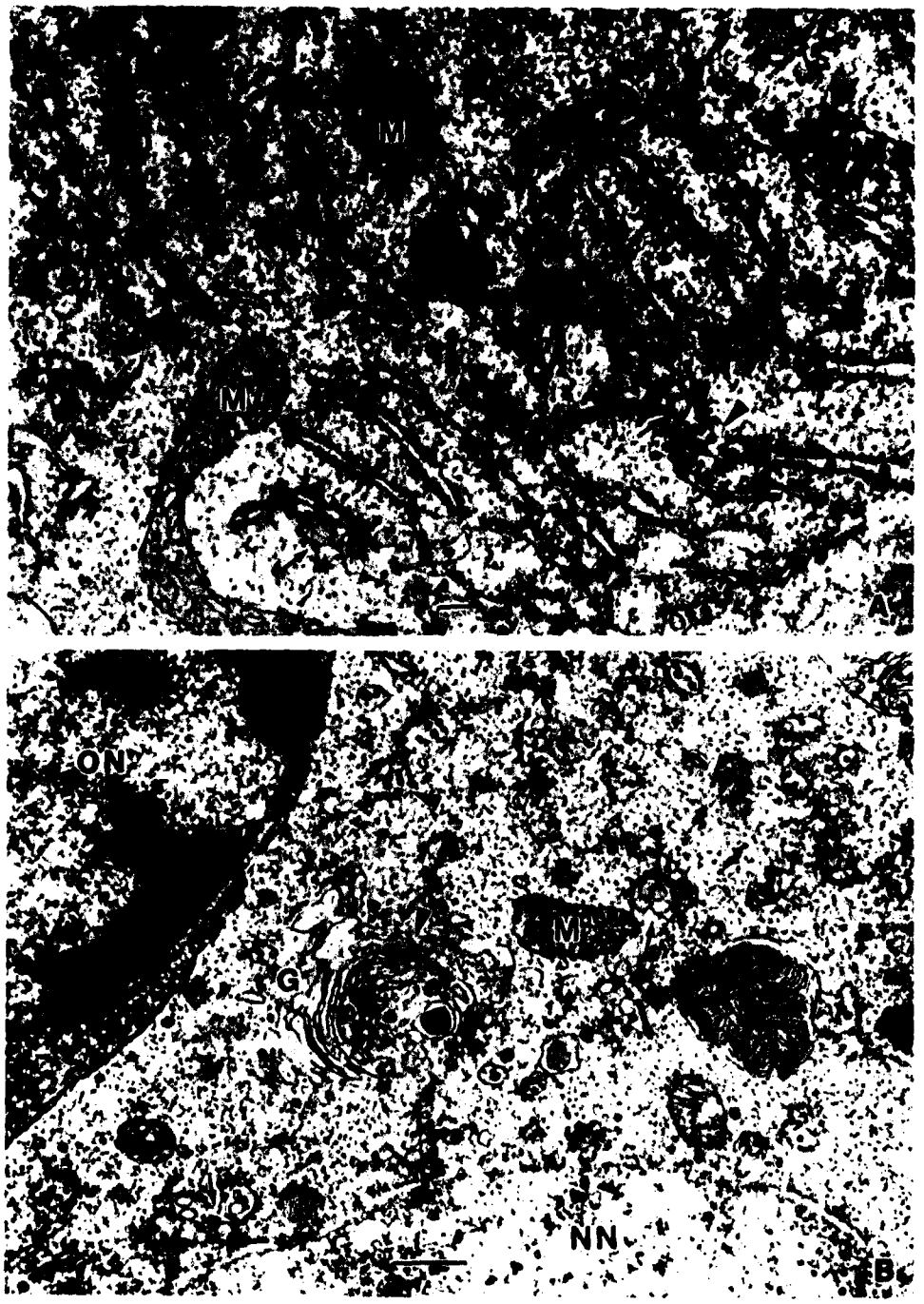

Fig. 3. Viral replication in the cisternae of RER and the Golgi apparatus of neurons in a mouse of the IC group. A. Hypertrophic RER of a neuron showing assembling virions (arrowheads) and characteristic ER vesicles (arrows) in the cisternae. M, mitochondrion. Bar, 100 nm. B. Virions (arrowheads) in the cisternae of fragmented RER and in the saccules of a Golgi complex (G). RER cisternae also contain ER vesicles (arrows). L, lysosome; M, mitochondrion; NN, neuronal nucleus; ON, oligodendrocytic nucleus. Bar, 500 nm.

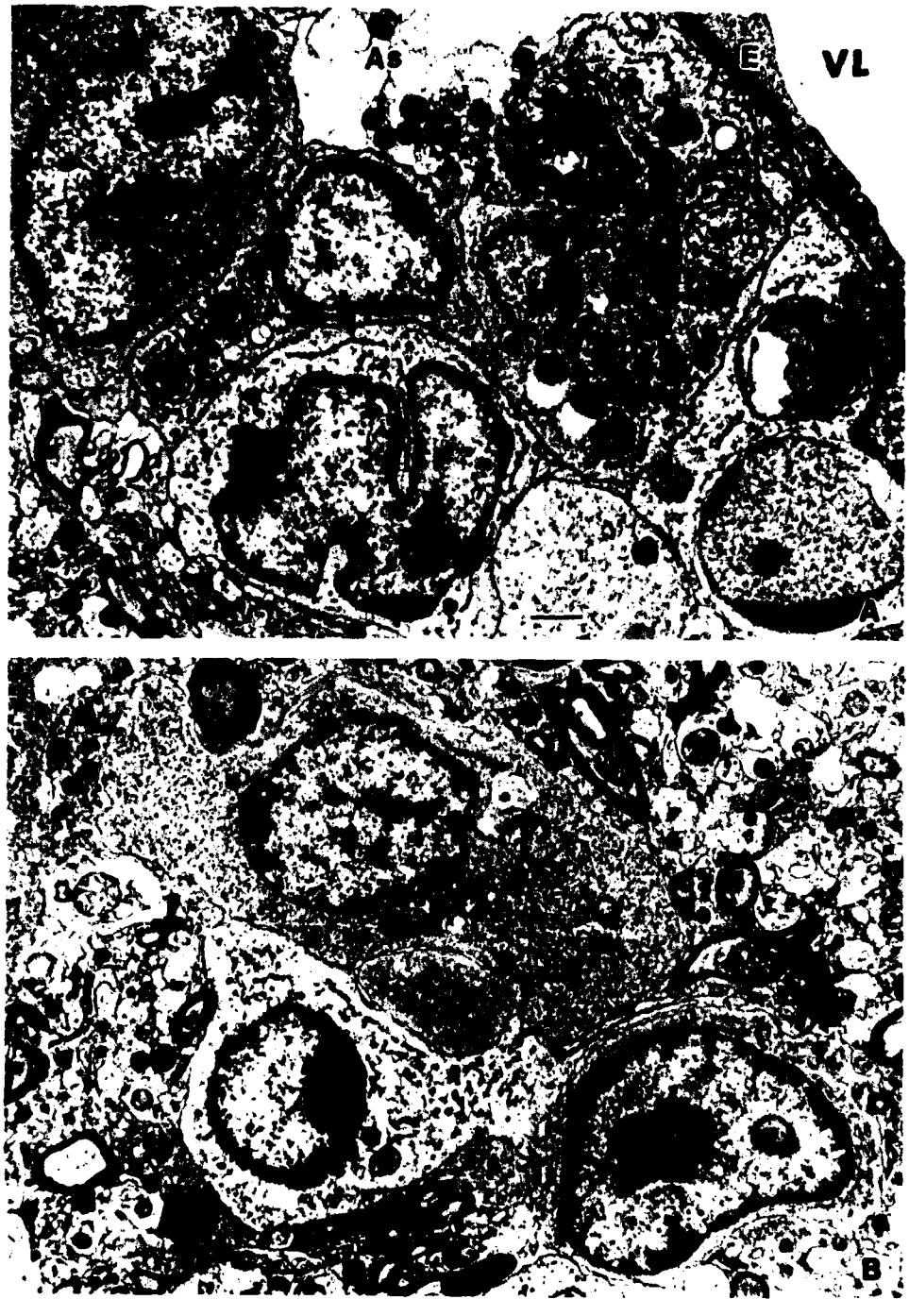

Fig. 4. Infiltrating mononuclear cells in the cerebral cortex of a mouse in the IP group. Bar. 1 μm. A. Mononuclear cells forming perivascular cuffing. As. swollen astrocytic foot-pad; E. vascular endothelium; VL. vascular lumen. B. Mononuclear cells in the neuropil.

these neurons appeared to be round, empty spaces with the nucleus in the centre. Fluffs and streaks of cytoplasmic material were recognizable contiguous to the nucleus or in the empty space. Neuronal destruction and neuronophagia were also commonly seen. Neuronal degeneration and necrosis were particularly prominent in the perivascular areas where marked oedema and leucocytic infiltration were present (Fig. 2D).

Electron microscopy

Ultrastructural changes of the mouse brains in intracerebrally inoculated mice were essentially the same as those reported pre-

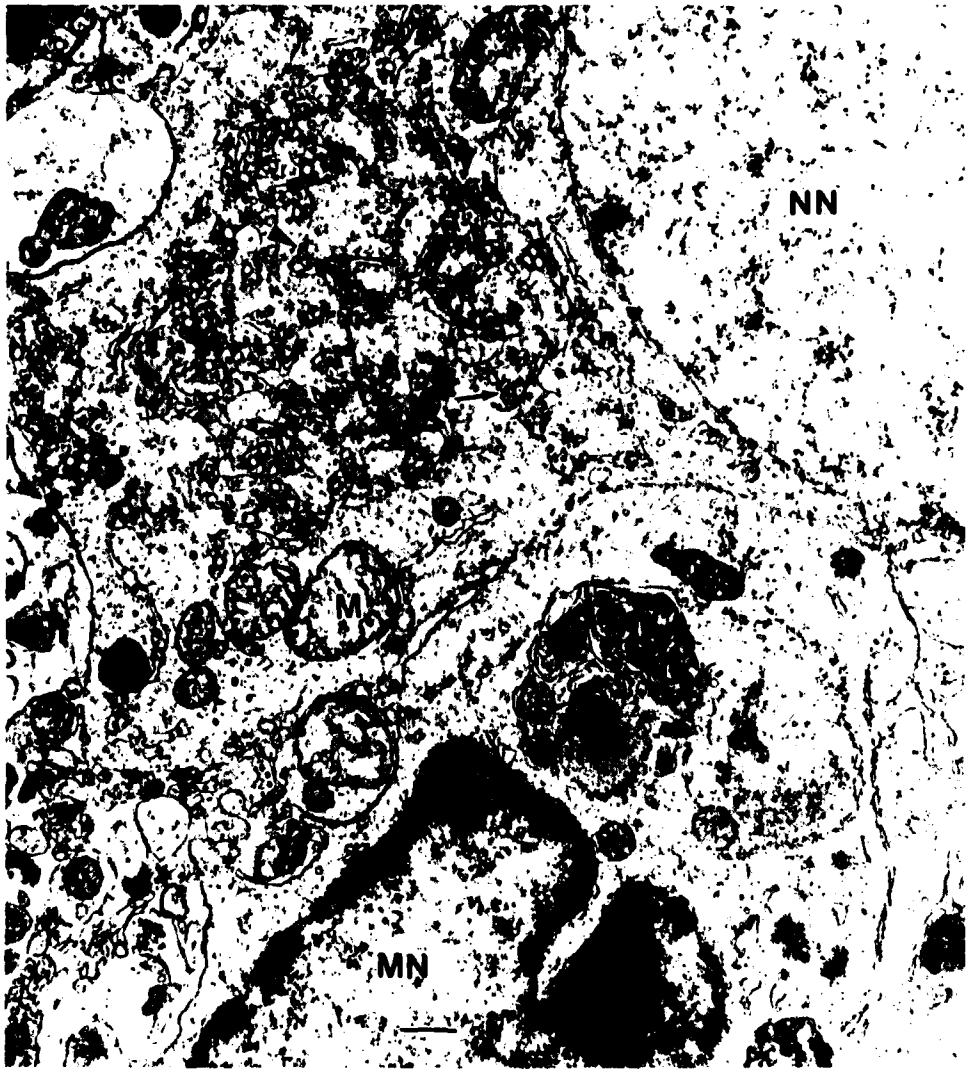

Fig. 5. An infected neuron in direct contact with a phagocytic mononuclear cell in the brain of a mouse in the IP group. Note clumping of RER segments in the neuronal perikaryon, whose irregularly dilated cisternae contain virions (arrowhead) and ER vesicles (arrow). L, lysosomes; M, mitochondrion; MN, mononuclear cell nucleus; NN neuronal nucleus. Bar, 500 nm.

viously (Hase *et al.* 1990). A majority of neurons showed multiple assembling virions and characteristic endoplasmic reticulum (ER) vesicles in the cisternae of RER (Fig. 3A). Progeny virions were also distributed in the neuronal secretory channel including the

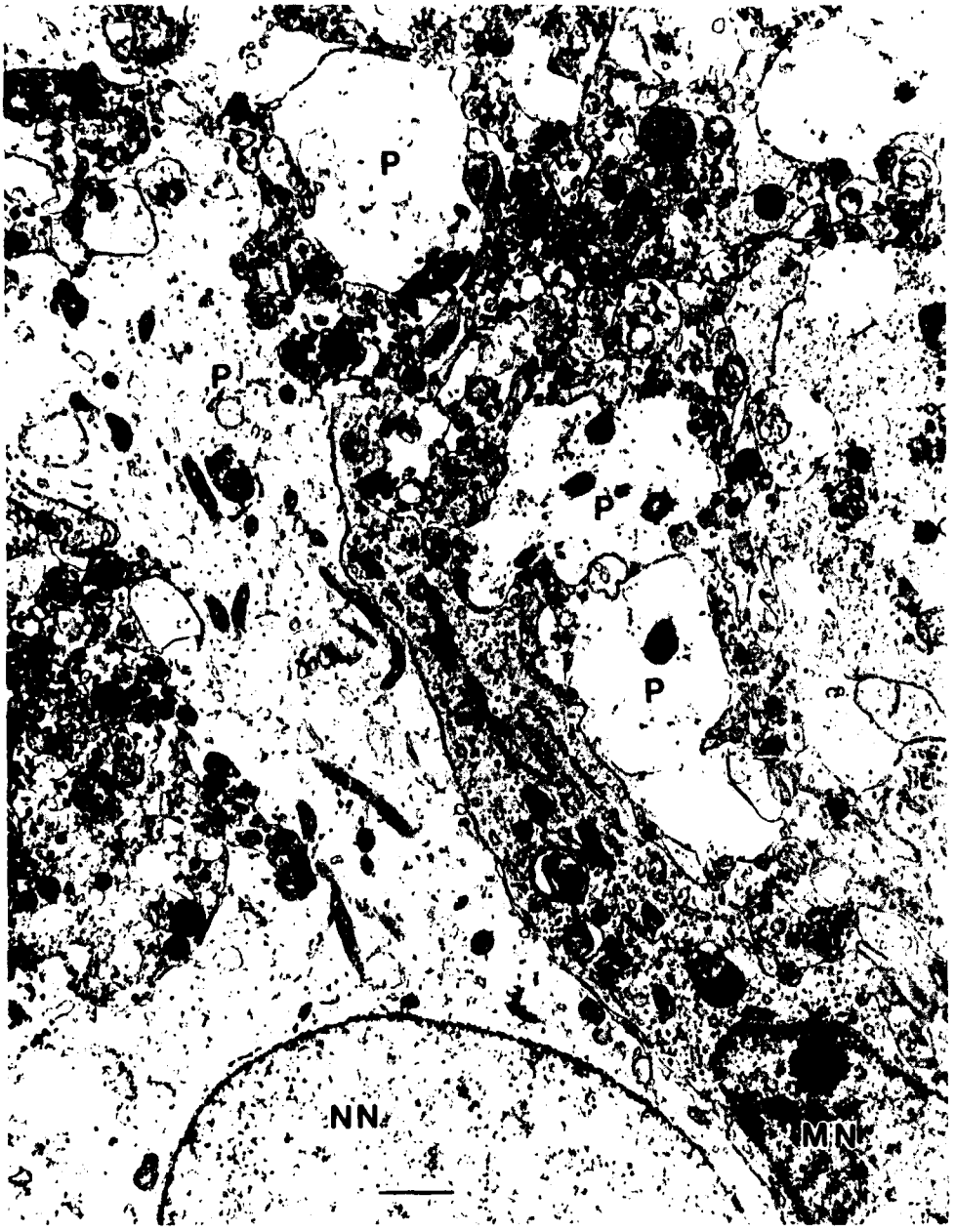

Fig. 6. Rarefaction of the cytoplasm and cytoplasmic processes (P) of a degenerating neuron in the brain of a mouse in the IP group. Note that a mononuclear cell closely apposes to the neuron. MN, mononuclear cell nucleus; NN, neuronal nucleus. Bar, 1 μm.

Golgi saccules and various transport vesicles (Fig. 3B). The distribution pattern of virions in neurons revealed trans-type maturation of JE virus (Hase *et al.* 1987, 1989). Accordingly, neurons showed varying degrees of hypertrophic or degenerative changes of RER and of degenerative changes of the Golgi apparatus in connection with JE virus growth in these organelles. Neuronal disintegration was rarely observed. Microglial cells and infiltrating mononuclear cells were not frequently encountered.

In the mouse brains of the IP + sIC and IP groups, many mononuclear and polymorphonuclear leucocytes were found, particularly in perivascular areas (Fig. 4A,B). Some

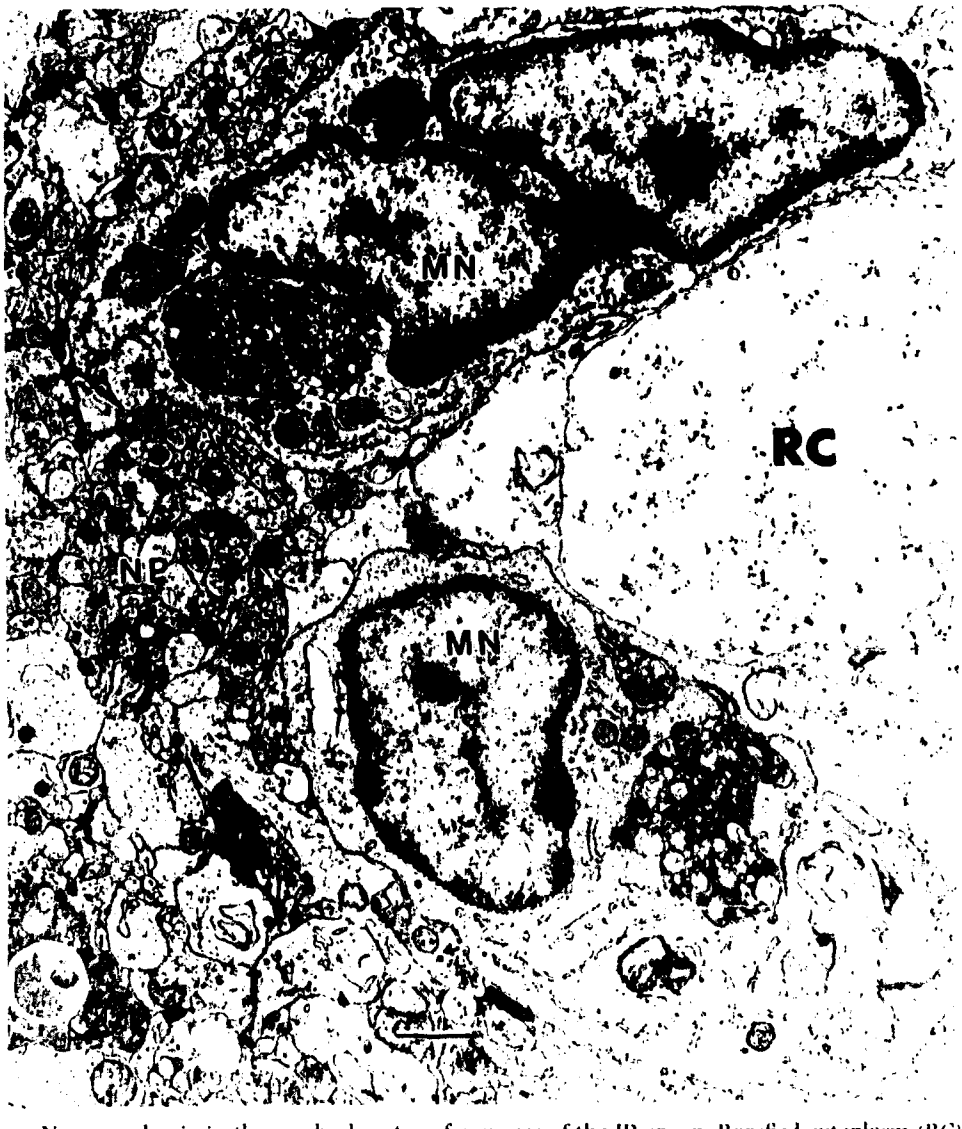

Fig. 7. Neuronophagia in the cerebral cortex of a mouse of the IP group. Rarefied cytoplasm (RC) of a degenerated neuron is surrounded by phagocytic mononuclear cells. MN, mononuclear cell nucleus; NP, neuropil. Bar, 1 μm.

of them showed prominent lysosomes that contained phagocytized cell and tissue debris. RER and the Golgi apparatus of these cells showed a normal appearance; no evidence was found that JE virus replicated in these cells. We also failed to find any evidence that JE virus replicated in the vascular endothelium and perivascular cells. In these groups, neurons that showed hypertrophic RER containing multiple assembling virions in its cisternae were rather infrequently encountered. Instead, many neurons showed in their perikaryon clumped RER segments whose cisternae contained ER vesicles and small numbers of virions (Fig. 5). Saccules of fragmented Golgi complexes also contained virions. Phagocytic cells that contained prominent lysosomes often apposed directly to infected neurons (Fig. 5). As RER and the Golgi apparatus of JE virus-infected neurons degenerated and dissolved, the neuronal cytoplasm showed an increasingly rarefied appearance, losing not only those organelles but also other cytoplasmic components such as free ribosomes, mitochondria, neurofilaments and microtubules, and neurosecretory vesicles (Fig. 6). The cytoplasmic rarefaction of infected neurons extended into the cytoplasmic processes including axons and dendrites. Disintegrating neurons having vacuous cytoplasm were often surrounded by mononuclear phagocytic cells for neuronophagia (Fig. 7).

Discussion

In the infection study of the three groups, all the mice of the IC group died rapidly in about 5 days p.i.; almost all the mice of the IP + sIC group eventually died between days 8 and 12; and only a certain percentage of the mice of the IP group died between days 12 and 16. Since peripheral inoculation into adult mice with JE virus does not cause 100% mortality, the combination of peripheral inoculation of JE virus and sham intracerebral inoculation has been used experimentally to increase the susceptibility of mice to the CNS infection (Yu *et al.* 1981; Eckels *et al.* 1988). Apparently, in this study, the concurrent sIC inoculation of mice at the time of IP inoculation with JE virus greatly facilitated viral invasion into the CNS. Although both the neural route, especially the olfactory route (Monath *et al.* 1983), and the haematogenous route (Johnson & Mims 1968) have been suggested, the mechanism by which neurotropic flaviviruses invade the CNS remains controversial. The present findings seem to suggest that a fresh wound created in the brain by sham intracerebral inoculation at the time of peripheral virus inoculation potentiates virus invasion into the CNS and, thus, support the haematogenous route of viral invasion. In nature, for reasons not understood, only extremely small percentages of JE virus-infected animals and humans manifest functional damage to the CNS (Webb & Smith 1966; Halstead 1981). It is conceivable, therefore, that as a rule the blood–brain barrier of animals and humans prevents viral invasion into the CNS and that JE virus gains entrance through some type of breach that is present in the barrier at the time of viral transmission. This breach might possibly be created by concurrent conditions such as trauma, allergic or inflammatory lesions, or toxic or nutritional damage to the vascular endothelial lining. In this respect, Kobiler *et al.* (1990) have reported that a variant of West Nile virus, WN-25 strain, that lacks neuroinvasiveness can be made neuroinvasive by concurrent administration of a reagent known to induce a breach in the blood–brain barrier such as CO_2, glycerol, mannitol, dimethoxysulphoxide (DMSO), or sodium dodecyl sulphate (SDS).

The essential feature of JE virus infection of the CNS of mammalian hosts is vigorous viral replication within the cellular secretory system including RER and the Golgi apparatus of neurons, with resultant damage to these cytoplasmic organelles (Hase *et al.* 1990). In the mouse brains of the IC group, this damage is the major morphological change that is recognizable only by the electron microscope. The absence of inflammatory and tissue-destructive changes in the

mouse brains of this group indicates that the extensive viral replication in neurons of the CNS was the direct cause of death. The same pathological changes are present in the mouse brains of the IP + sIC and IP groups; however, the neuronal changes of these groups were accompanied by prominent inflammatory reactions that are easily recognizable by light microscopy. Histological examinations of mouse brains in these groups showed infiltration of mononuclear and polymorphonuclear cells in the leptomeninges and brain tissue, with frequent formation of perivascular cuffing. Characteristic cytoplasmic rarefaction of neurons seen in histological preparations may reflect disappearance of cytoplasmic organelles seen in electron microscopical preparations. Neuronal degeneration and necrosis, and neuronophagia were frequently encountered. The histological changes of the brains of the peripherally inoculated mice, therefore, seem closely to simulate those in human cases of Japanese encephalitis. We also studied the mouse brains of the IP + sIC and IP groups that survived. No pathological lesions were found in the brains of these mice by light and electron microscopy. This indicated that JE virus never invaded the CNS of the mice that survived.

The present study demonstrates that the basic pathogenic mechanism of JE virus infection of the mouse CNS is viral growth within neurons, regardless of the route by which the virus is introduced. On the other hand, only the mice peripherally inoculated with JE virus showed marked inflammatory reaction. It has been reported that peripherally inoculated JE virus first proliferates in the peripheral tissue, causing secondary viraemia (Huang & Wong 1963). In this study, it is difficult to determine whether the absence or the presence of inflammatory reaction depended on the time course of infection or the inoculation site. Occasionally, we encountered IC inoculated mice that survived almost as long as IP + sIC inoculated mice; however, the brains of the former mice showed far less inflammatory changes than the brains of the latter mice. Therefore, it is possible that IC inoculation of JE virus *per se* does not effectively stimulate the host immune response; perhaps, the blood–brain barrier works both ways in that it prevents peripherally inoculated virus from reaching the CNS and IC inoculated virus from reaching the peripheral immunological organs. In contrast, peripherally inoculated virus may stimulate host immunological response effectively to cause inflammatory reactions in the brain. Webb and Smith (1966) have proposed that virus-induced encephalitis is the result of host immunological response directed against virus-infected neurons, suggesting that direct viral cytopathic effects on neurons and inflammatory reactions are two separate processes. The present study seems to support their idea in that the neuronal changes in the mouse brains of the IC group represented direct viral action to neurons and the inflammatory reaction in the mouse brains of the IP + sIC and IP groups represented host immunological response to virally infected neurons. Whether the inflammatory reaction in the JE virus-infected mouse brains is beneficial or deleterious to host animals is a difficult question to answer. The presence of host immunological response may prevent further spread of virus among neurons. On the other hand, cytotoxic and phagocytic activities on virally infected neurons by immunologically activated lymphocytes and macrophages can cause serious problems, since neurons are individually unique cells that are irreplaceable. In JE virus infection of spider monkeys, Nathanson and Cole (1970) have shown that immunosuppressed animals had higher death rates, although histological signs of inflammation were reduced. In mice, JE virus infection of neurons in the CNS is a rapidly fatal disease; therefore, any prolongation of the disease course by the concurrent immunological response may be regarded to be a beneficial effect.

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